Inhibition of calcineurin phosphatase promotes exocytosis of renin from juxtaglomerular cells

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To examine the role of the calcium/calmodulin-dependent phosphatase calcineurin in regulation of renin release, we assayed exocytosis using whole-cell patch clamp of single juxtaglomerular cells in culture. The calcineurin inhibitor, cyclosporine A (CsA), significantly increased juxtaglomerular cell membrane capacitance, an index of cell surface area and an established measure of exocytosis in single-cell assays. This effect was mimicked by intracellular delivery of a calcineurin inhibitory peptide, the calcium chelator ethylene glycol tetraacetic acid (EGTA), or the calmodulin inhibitor W-13. Simultaneous exposure to EGTA and CsA had no additive effect. The protein kinase A (PKA) blocker RpcAMPs had no effect on the CsA-induced increase in membrane capacitance. Intra- and extracellular application of tacrolimus did not alter membrane capacitance. A calmodulin antagonist (calmidazolium) and CsA, but not tacrolimus, significantly stimulated renin release from cultured juxtaglomerular cells. Juxtaglomerular cells expressed the calcineurin isoforms A-β and A-γ but not A-α. Plasma renin concentrations (PRCs) were not different in wild-type, calcineurin A-α, or A-β knockout mice but increased after CsA treatment of the A-α knockout, while renin mRNA was suppressed. We conclude that calcineurin and calcium/calmodulin suppress exocytosis of renin from juxtaglomerular cells independent of PKA.

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Renin is stored and released by juxtaglomerular granular (JG) cells in the distal part of the afferent renal arterioles. The activity of the circulating renin–angiotensin–aldosterone system is determined by the secretion rate of active renin from JG cells. In most secretory cells, a rise in the intracellular concentration of calcium ($[Ca^{2+}]_i$) initiates or maintains secretory activity, but a rise in $[Ca^{2+}]_i$ is associated with inhibition of renin release: calcium channel blockers stimulate renin release,[1] depolarization activates calcium channels and inhibits renin release in single JG cells,[2] cell-permeable calcium chelators increase renin release,[3] liberators of calcium from intracellular stores inhibit renin release,[4] and calcium-mobilizing hormones suppress renin secretion.[5] Calcium inhibits adenylate cyclase, isoforms 5 and 6, in JG cells, and thereby impairs the ability of the cells to increase cyclic adenosine monophosphate (cAMP) and renin secretion.[3,6] A second relevant target for calcium in JG cells is the calcium-dependent protein calmodulin, as inhibitors of calmodulin increase renin release from *in vitro* preparations.[7–9] Because adenylate cyclases 5 and 6 are not sensitive to calmodulin,[10] an additional calcium-regulated, inhibitory, renin secretory pathway is likely to exist. This notion is supported by the observation that activation of voltage-gated calcium channels by depolarization prevents cAMP-mediated exocytosis of renin in single JG cells, which indicates that calcium may inhibit renin secretion independently of formation of cAMP.[2] To explain this effect, the calcium/calmodulin-stimulated protein phosphatase calcineurin appears relevant. Calcineurin inhibitor immunosuppressants increase plasma renin concentration (PRC)[11–14] and cyclosporine A (CsA) stimulates renin secretion from JG cell cultures in a cAMP-independent manner.[15] Calcineurin is a heteromultimeric protein, and the catalytic A subunit exists in three different isoforms encoded by distinct genes.[16,17] In this study we hypothesized that (1) catalytic calcineurin subunits are expressed in renin-secreting JG cells, (2) calcineurin activity suppresses exocytosis of renin, and (3) calcium inhibits exocytosis of renin through calmodulin-dependent calcineurin activity. These hypotheses were analyzed in single JG cells with the whole-cell patch-clamp approach. This technique enables real-time recordings of

exocytosis independent of systemic factors and it has been extensively validated on single JG cells.[18,19] Electrophysiological data were corroborated by studies of renin release from primary cultures of renal cortical cells enriched in JG cells. PRC was measured in wild-type mice and mice with disruption of calcineurin A-α and A-β isoforms with and without CsA treatment.

RESULTS

The whole-cell recording was obtained in single JG cells isolated from 32 preparations. JG cells showed outward current rectification at positive membrane potentials and very limited net currents between −30 and 0 mV.[18,19] The JG cells had an average membrane capacitance (C_m) value of 2.00 ± 0.15 pF ($n = 33$). Mean C_m values determined at $t = 0$ in each experimental series were not significantly different. Under control conditions, C_m was stable, and the current–voltage relationship was not affected by the whole-cell mode within the time of recording (10 min).

Effect of calcineurin antagonists on juxtaglomerular cell membrane capacitance (C_m) and renin secretion

Cyclosporine A (5 μmol/l) increased significantly JG-cell C_m ($P < 0.05$, Figure 1a and e). Whole-cell currents did not change (Figure 1a). Tacrolimus (0.1 μmol/l) added in the bath had no effect on C_m (Figure 1b and e). When added directly to the JG-cell cytoplasm, tacrolimus (0.1 μmol/l) did not change C_m (Figure 1c and e, $n = 5$). A calcineurin-inhibitory peptide evoked a significant increase in C_m when dialyzed into single JG cells (Figure 1d and e). Tacrolimus and calcineurin inhibitory peptide had no effect on whole-cell currents (data not shown). CsA significantly stimulated renin release from JG cells ($P < 0.01$, Figure 2a). Tacrolimus had no effect on renin release from JG cells (Figure 2b). A combined application of forskolin and CsA significantly augmented renin secretion from JG cells compared with CsA and forskolin alone (Figure 2c). Forskolin stimulated renin release significantly compared with control in all series (Figure 2a–c, $P < 0.01$).

Expression of catalytic calcineurin A subunit isoforms and calcineurin inhibitor binding proteins

Calcineurin A-β (CnA-β) and CnA-γ, but not CnA-α, were detected in preglomerular rat microvessels (Figure 3a). The microvessels and renin-producing As4.1 cells expressed binding proteins for CsA and tacrolimus (Figure 3b). In patch pipette-sampled native JG cells, CnA-α was at the limit of detection, whereas CnA-β and CnA-γ were readily amplified (Figure 3c). As4.1 cells expressed CnA-β and CnA-γ but not CnA-α (Figure 3d). CnA-α and CnA-γ primers did not cross-amplify the other calcineurin isoform (Figure 3e and f). Amplified products were 100% homologous to GenBank sequences. Amplification was achieved only in the presence of reverse transcriptase and cDNA (Figure 3).

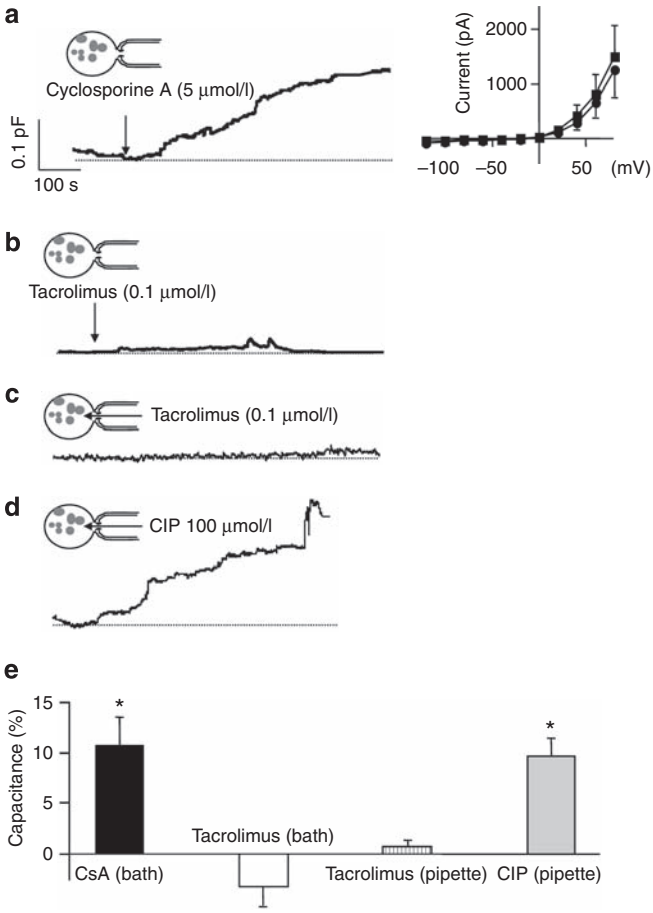

Figure 1 | Effect of calcineurin inhibitors on JG cell membrane capacitance (C_m) in single rat JG cells. Schematic cell models in (**a**–**d**) show the experimental approaches with intra- and extracellular application of inhibitors. (**a**) Left: The trace from a single JG cell shows the effect of superfusion with cyclosporine A (CsA; 5 μmol/l) on C_m. Right: The average current–voltage (I-V) relationship was measured immediately after the whole-cell configuration was obtained in single JG cells (filled circles). The measurement was repeated 10 min after application of CsA (filled squares). (**b**, **c**) The C_m traces from single JG cells show the effect of (**b**) superfusion and (**c**) intracellular dialysis with tacrolimus (0.1 μmol/l) on C_m. (**d**) The C_m recording from a single JG cell shows the effect of intracellular dialysis with calcineurin inhibitory peptide (CIP, 100 μmol/l). (**e**) Average changes of C_m in response to CsA, extra- and intracellular tacrolimus, and CIP ± s.e. *$P < 0.05$ at $t = 0$ vs $t = 10$ min ($n = 4$–6).

Effect of protein kinase A (PKA) inhibitor and a calcium chelator on the capacitance-response to CsA in juxtaglomerular cells

The PKA inhibitor, RpcAMPs (5 μmol/l), did not alter the CsA-mediated increase in C_m (Figure 4a). When $[Ca^{2+}]_i$ was lowered by dialysis of single JG cells with ethylene glycol tetraacetic acid (EGTA; 0.1 mmol/l), C_m increased promptly (Figure 4b). When EGTA and CsA were added simultaneously to single JG cells, there was no further augmentation of C_m by the combination, compared with CsA or EGTA alone (Figure 4c). Whole-cell currents did not change by any of these maneuvers (data not shown).

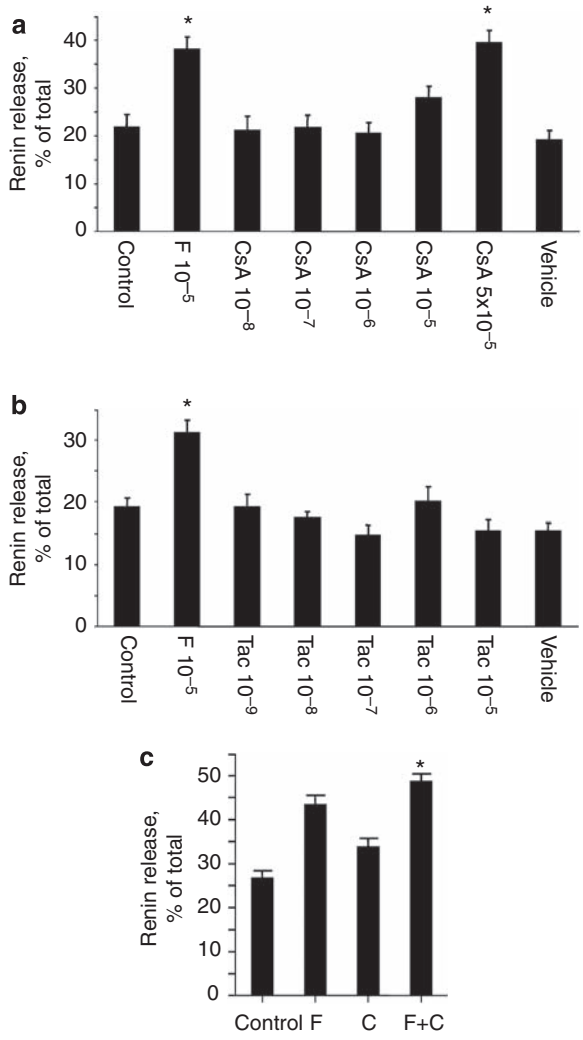

Figure 2 | Renin release experiments. (**a**) Renin release from primary cultures of renal cortical cells enriched in JG cells in response to cyclosporine A in mol/l (CsA, $*P<0.01$, $n=16$ from four different preparations, at 20-h incubation). The figure shows renin release as the percentage of total renin content. Forskolin (F) served as a positive control. $*P<0.01$ compared with control. (**b**) Renin release from primary cultures of renal cortical cells enriched in JG cells in response to tacrolimus in mol/l (Tac). The figure shows renin release as the percentage of total renin content. Forskolin (F) served as a positive control. $*P<0.01$, $n=12$ from three different preparations. (**c**) Renin release from primary cultures of renal cortical cells enriched in JG cells in response to forskolin (F, 10^{-5} mol/l), CsA (C, 10^{-4} mol/l), and combined forskolin and cyclosporin A (F + C). Renin release is shown as the percentage of total renin content. $*P<0.05$ compared with all other conditions; $n=11$ from three different preparations.

Effect of calmodulin antagonist on JG cell membrane capacitance and renin secretion

Intracellular dialysis with the calmodulin antagonist W-13 (100 µmol/l) increased C_m significantly (Figure 5a). The stimulatory effect of W-13 was not modified by concomitant dialysis of single JG cells with the PKA inhibitor RpcAMPs (Figure 5b). The cell-permeable calmodulin antagonist calmidazolium stimulated renin release significantly from primary cultures of JG cells. (Figure 5c).

Effect of cyclosporine A (CsA) on plasma renin concentration (PRC) and renin mRNA level in wild-type mice and mice with targeted disruction of calcineurin isoforms

Basal PRC was not significantly different in CnA-$\beta^{-/-}$ and CnA-$\alpha^{-/-}$ mice compared with wild-type littermates (Figure 6a). Treatment with cyclosporine for 4 days did not alter PRC significantly in wild-type mice ($P<0.07$, Figure 6a) and in CnA-$\beta^{-/-}$ mice ($P<0.2$), but led to a significant increase in CnA-$\alpha^{-/-}$ mice ($P<0.01$, Figure 6a). Renin mRNA level (Figure 6b) was not significantly different between wild-type and CnA-$\alpha^{-/-}$ mice. CsA treatment significantly suppressed renin mRNA level in kidneys from wild-type mice and CnA-$\alpha^{-/-}$ mice (Figure 6b). In renin-expressing As4.1 cells, CsA suppressed renin mRNA level after a 20-h incubation, whereas forskolin significantly increased renin mRNA abundance (Figure 6c).

DISCUSSION

In this study, individual JG cells were used for patch clamp when they showed the characteristic current–voltage relation.[2,18,19] An increase in C_m indicates net addition of membrane to the cell surface and is a well-established measure of exocytosis at the single-cell level.[20] Chelation of intracellular calcium and inhibitors of calmodulin and the serine/threonine phosphatase calcineurin all stimulated exocytosis of renin. Exocytosis of renin subsequent to exposure to CsA and calmodulin antagonist was not dependent on cAMP/PKA and was not accompanied by changes in membrane currents, which are stimulated consistently by cAMP.[18] The macrolide calcineurin inhibitor tacrolimus had no effect on C_m and renin secretion.

Increases in C_m of ~10%, as observed in this study, corresponds to release of app.10 granules from mouse JG cells[18] and the data are compatible with the notion that calcineurin is a target protein for calcium/calmodulin in a cAMP-independent pathway that suppresses exocytosis of renin granules (Figure 7). This is consistent with previous data showing that calmodulin antagonists and calcineurin inhibitors do not alter cAMP in JG cells.[7,15] The present data show directly at the single-cell level that intracellular calcium constitutes a 'brake' on renin exocytosis. Calcineurin is calcium-calmodulin dependent, but multiple signaling pathways are likely altered through chelation of calcium. Thus, chelation of intracellular calcium by cell-permeable 1,2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid raises cAMP in JG cell cultures, probably through calmodulin-independent stimulation of adenylate cyclases 5 and 6, and a similar response could contribute to the observed rise of C_m in single JG cells after EGTA.[3] The *in vitro* data imply that calcineurin phosphatase is constitutively active to suppress renin exocytosis. The prolonged treatment of wild-type mice with CsA *in vivo* tended to increase PRC, which is similar to data from rats in which a 7-day treatment protocol resulted in PRC changes at

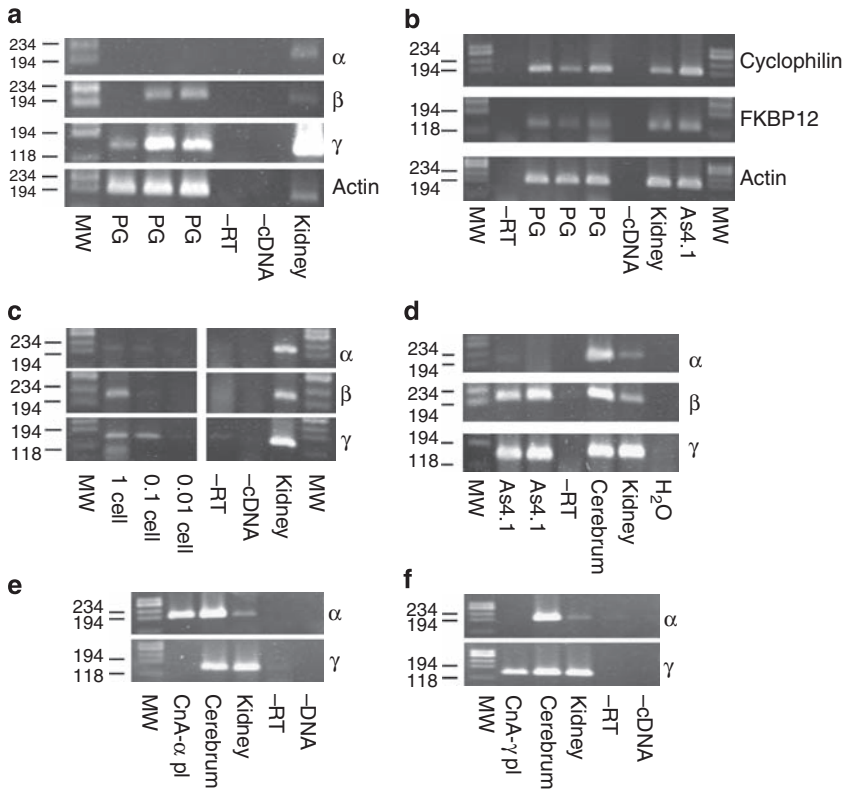

Figure 3 | Expression of catalytic calcineurin isoforms and calcineurin inhibitor immunosuppressant (CNI) binding proteins in preglomerular microvessels and single JG cells using PCR. In all experiments, negative controls were omission of reverse transcriptase (−RT) and cDNA (−cDNA) and molecular size marker (MW) was ϕX174DNA/*Hae* III fragments. (**a**) Amplification of cDNA from microdissected preglomerular (PG, $n = 3$) microvessels for calcineurin A (CnA)-α, CnA-β, and CnA-γ. CnA-α was not detected. (**b**) Amplification of cDNA from PG microvessels ($n = 3$) for cyclophilin and FKBP12. Cyclophilin and FKBP12 were readily amplified with the expected molecular size (186 and 134 bp, respectively) from microvessels and renin-producing As4.1 cells. (**c**) Amplification of serially diluted cDNA from single, sampled JG cells for CnA isoforms. CnA-β and CnA-γ were readily detected. (**d**) Amplification of cDNA from two preparations of As4.1 cells for CnA isoforms. CnA-β and CnA-γ were readily detected. (**e**) Amplification of CnA-α and CnA-γ with plasmid template (pl) carrying the full sequence of murine CnA-α. Only CnA-α was readily amplified. Expected molecular size: 187 + 15 bp, + 15 refers to the addition of restriction sites. (**f**) Amplification of CnA-α and CnA-γ with plasmid template (pl) carrying the full sequence of calcineurin A-γ. Only CnA-γ was readily amplified. Expected molecular size: 117 + 15 bp.

the limit of significance.[21] This difference between *in vitro* and *in vivo* sensitivity to cyclosporine is likely caused by the different time of intervention and the highly redundant regulation of renin secretion at the systemic level. It has been discussed whether the stimulatory effect of CsA on renin release *in vivo* is due to a direct effect on JG cells or it is secondary to hemodynamic changes induced by CsA.[22,23] These data show that CsA directly and acutely stimulates exocytosis of renin, consistent with observations in JG cell cultures by Kurtz *et al.*[15] The concentrations of CsA and tacrolimus applied to the JG cells are clinically relevant as plasma concentrations observed at nadir and peak in patients typically are in the micromolar range (cyclosporin) and nanomolar range (tacrolimus).[24] The main intracellular target of both CsA and tacrolimus is calcineurin, but the two drugs showed differential effects on renin release and C_m changes. At the systemic level, differential effects on cardiovascular parameters have been reported. Klein *et al.*[25] showed increased blood pressure, increased renal vascular resistance, and decreased renal blood flow in response to CsA in healthy adult volunteers, whereas tacrolimus induced no such changes. Similar findings have been obtained in rats.[22,23] The lack of effect of tacrolimus was not caused by impaired cellular uptake or absence of binding protein.[26,27] Differential effects of blockers on distinct calcineurin isoforms could be involved. Thus, three separate genes encode the catalytic subunit of calcineurin, CnA-α, CnA-β, and CnA-γ. CnA-α is developmentally downregulated in rodent kidney.[28] In agreement, CnA-α was at the limit of detection in preglomerular vasculature and JG cells, and PRC increased significantly by CsA in CnA-$\alpha^{-/-}$ mice. This indicates a minor role of this isoform in the control of renin secretion and mRNA. CnA-β and CnA-γ were expressed in the renal vasculature and in single JG cells. CnA-β is of major importance for T-lymphocyte function[29] and has significant cardiovascular effects,[30] but its deletion did not alter basal PRC. This could be caused by redundant, compensatory effect of intact CnA-γ. CnA-γ has previously only been described in testis and brain tissue[17] and targeted deletion has not yet been achieved. If there is redundancy between CnA-γ and CnA-β in the control of renin, a preferential effect of

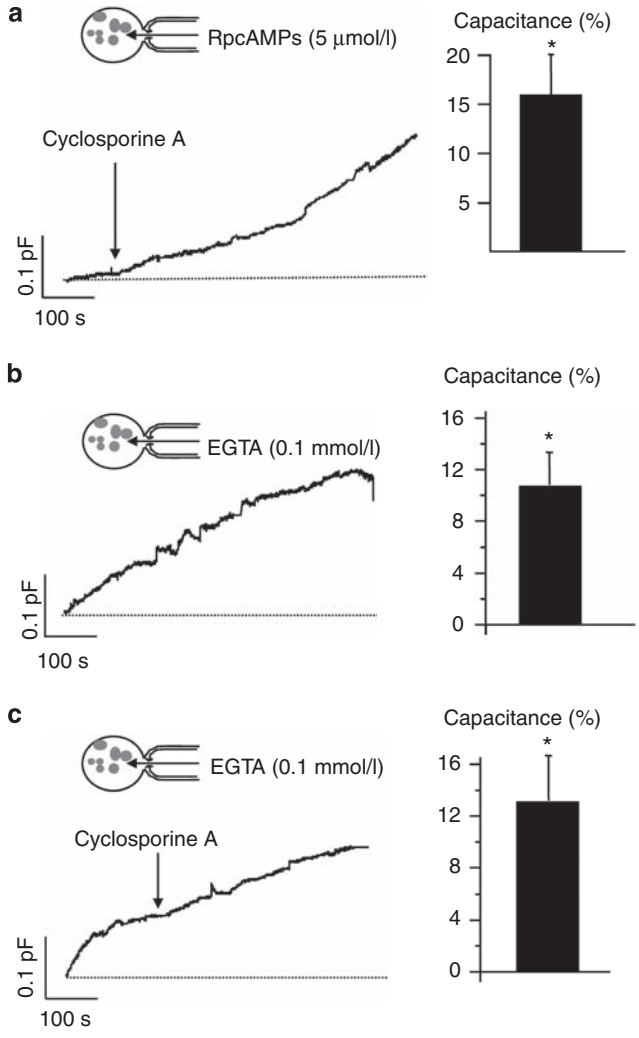

Figure 4 | Schematic cell models showing the experimental approaches with combined intra- and extracellular application of drugs. (**a**) Left: The C_m trace from a single JG cell shows the effect of combined cyclosporine A (CsA; 5 μmol/l) and dialysis of the cell with the protein kinase A (PKA) inhibitor RpcAMPs (5 μmol/l). Right: average changes of C_m in response to CsA and RpcAMPs. *$P<0.05$ at $t=0$ vs $t=10$ min ($n=4$). (**b**) Left: A C_m trace obtained in a single JG cell shows the effect of intracellular dialysis with ethylene glycol tetraacetic acid (EGTA; 0.1 mmol/l). Right: average changes of C_m in response to EGTA. *$P<0.05$ at $t=0$ vs $t=10$ min ($n=4$). (**c**) Left: The C_m trace from a single JG cell shows the effect of CsA (5 μmol/l) and concomitant dialysis of the cell with EGTA (0.1 mmol/l). Right: average changes of C_m in response to CsA and EGTA. *$P<0.05$ at $t=0$ vs $t=10$ min ($n=5$).

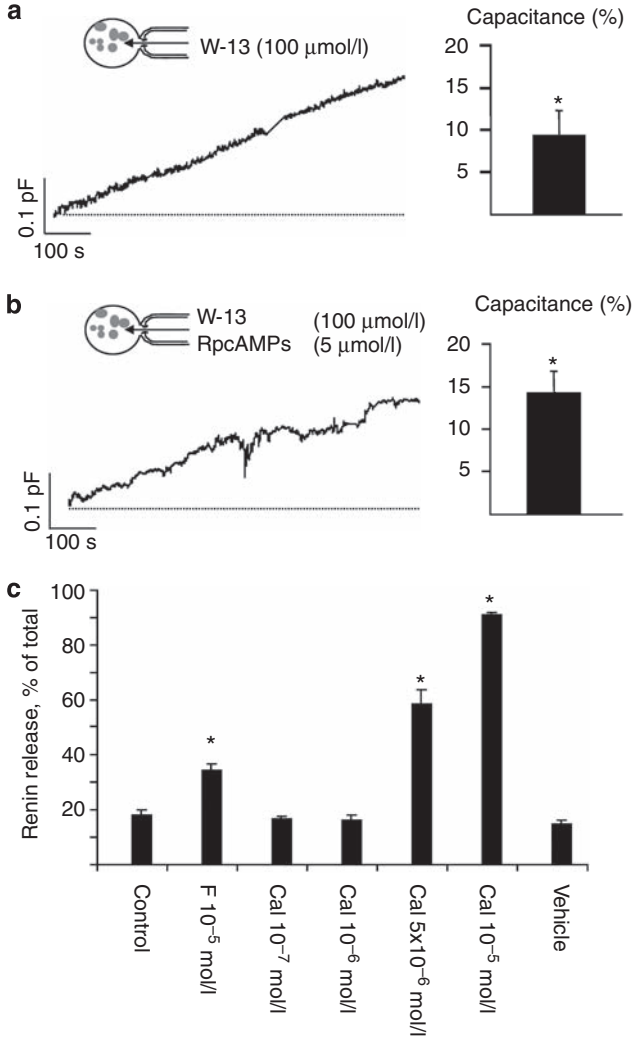

Figure 5 | Schematic cell models showing the experimental approach with intracellular application of inhibitors through the patch pipette. (**a**) Left: The C_m trace from a single JG cell shows the effect of intracellular application of the calmodulin inhibitor W-13 (100 μmol/l). Right: average changes in C_m in response to W-13. *$P<0.05$ at $t=0$ vs $t=10$ min ($n=4$). (**b**) Left: an original C_m trace from a single JG cell shows the effect of simultaneous intracellular application of the calmodulin inhibitor W-13 (100 μmol/l) and the protein kinase A (PKA) blocker RpcAMPs (5 μmol/l). Right: average changes in C_m in response to W-13 and RpcAMPs. *$P<0.05$ at $t=0$ vs $t=10$ min ($n=4$). (**c**) Renin release from renal cortical cells enriched in JG cells in response to a cell-permeable calmodulin inhibitor, calmidazolium (Cal, $n=16$ from four different preparations, *$P<0.01$). Forskolin (F) served as positive control, *$P<0.01$.

tacrolimus on one isoform could be a relevant explanation for the lack of effect on renin secretion by tacrolimus. These data show suppression of renin mRNA *in vivo* after treatment with CsA for 4 days and the effect was also found *in vitro*, which implies a direct effect that potentially could be mediated by the nuclear factor of activated T-cells transcription factors, which are expressed in vessels. We show a direct and differential effect of clinically used calcineurin inhibitors to stimulate exocytosis of renin from single JG cells in a cAMP/PKA-independent manner. Chelation of intracellular calcium and inhibition of calmodulin mimicked the effect of calcineurin inhibition at the JG-cell level. CnA-β and CnA-γ are likely targets for calcium/calmodulin in a pathway leading to suppression of renin exocytosis. Future studies should explore and identify molecular substrate targets that are dephosphorylated by calcineurin in JG cells.

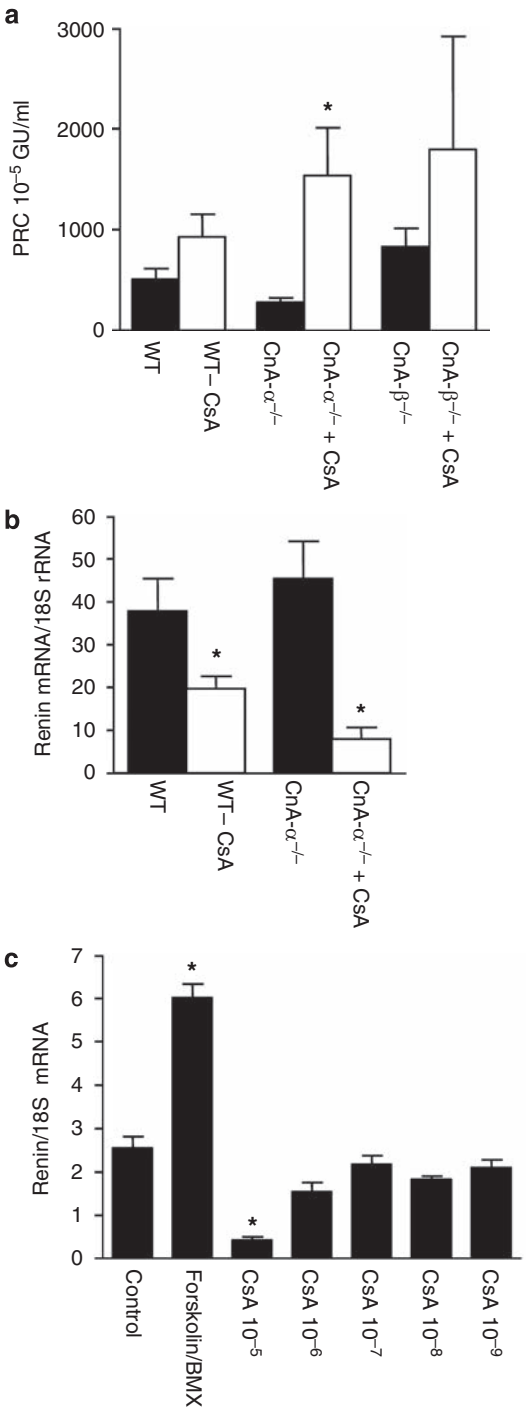

Figure 6 | Effect of calcineurin gene disruption and cyclosporine treatment in mice. (**a**) Columns show plasma renin concentration (PRC, 10^{-5} GU/ml) in wild-type (WT) C57Bl6 mice treated with vehicle ($n = 12$), WT mice treated with cyclosporine A (CsA, 20 mg/kg, 4 days, $n = 5$), calcineurin A-α-deleted mice (CnA-$\alpha^{-/-}$, $n = 7$), CnA-$\alpha^{-/-}$ mice treated with CsA (20 mg/kg, 4 days, $n = 5$), CnA-$\beta^{-/-}$ ($n = 8$), and CnA-$\beta^{-/-}$ treated with CsA (20 mg/kg, 4 days, $n = 5$). *$P < 0.01$. (**b**) Effect of cyclosporine (CsA) treatment on renin mRNA levels in kidney tissue from WT mice and CnA-$\alpha^{-/-}$ mice. *$P < 0.01$. (**c**) Effect of cyclosporine and forskolin/IBMX on renin mRNA levels in cultured As4.1 cells. *$P < 0.01$, $n = 7$.

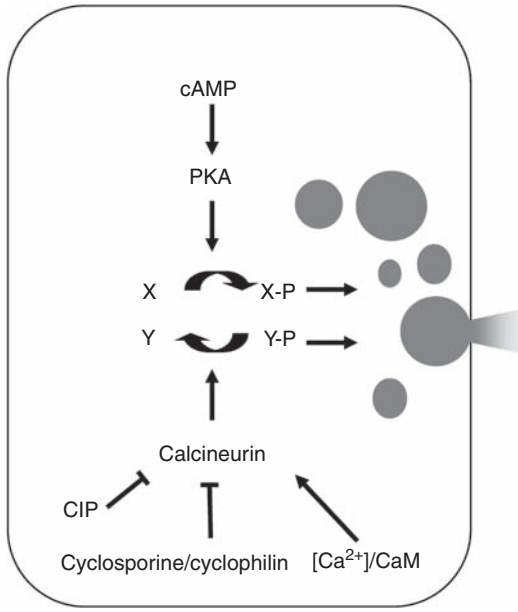

Figure 7 | Schematic drawing of a single juxtaglomerular granular cell that shows the pathways elucidated in this study. CaM, calmodulin; cAMP, cyclic adenosine monophosphate; CIP, calcineurin inhibitory peptide; -P, phosphate group; PKA, protein kinase A; X and Y, unknown substrate proteins.

MATERIALS AND METHODS

In vivo experiments

All procedures conformed to the Danish national guidelines for the care and handling of animals and the published guidelines from the National Institutes of Health. Male Sprague–Dawley rats (60–80 g) were housed at University of Southern Denmark, on a 12:12 h light:dark cycle and had free access to standard pathogen-free rat chow (Altromin-1310, Lage, Germany; Na^+ 2 g/kg and Cl^- 5 g/kg) and tap water. CnA-$\alpha^{-/-}$ and CnA-$\beta^{-/-}$ mice were bred in the animal facility at the Atlanta VA Medical Center following the guidelines of the institution animal care and use committee. Genotyping was performed as previously described.[31] Wild-type and CnA-$\alpha^{-/-}$ and CnA-$\beta^{-/-}$ mice were treated with cyclosporine 20 mg/kg per day by intraperitoneal injection once daily for 4 days. Mice were anesthetized with isoflurane and blood was sampled by cardiac puncture. Kidneys were rapidly frozen in liquid nitrogen.

Isolation of juxtaglomerular cells

JG cells were isolated from rat renal cortex as described.[19] A total of 40 rats were used. For patch clamp, cells were transferred to cover slips in RPMI-1640 medium. A specific condition was tested on not more than one cell from one preparation, and hence, for example, $n = 5$ means recordings from five single cells from five different animals. Renin secretion studies were performed as described.[18,19] As4.1 cells were grown according to the American Type Culture Collection instructions. As4.1 cell medium was replaced by Dulbecco's modied Eagle's medium containing 2% fetal calf serum at 24 h before experiments.

Patch-clamp experiments

The patch-clamp experiments were performed as described[18,19] with internal solution (in mmol/l): K-glutamate 135; NaCl 10; KCl 10;

$MgCl_2$ 1; 4,(2-hydroxyethyl)-1-piperazine ethane sulphonic acid-NaOH 10; magnesium-adenosine triphosphate 0.5; Na_2-guanosine triphosphate 0.3; osmolality was 303 mOsm/kg; pH 7.07 with or without RpcAMPs (5 μmol/l). External solution was 4,(2-hydroxyethyl)-1-piperazine ethane sulphonic acid-NaOH 10; NaCl 140; KCl 2.8; $MgCl_2$ 1; $CaCl_2$ 2; glucose 11; sucrose 10; osmolality was approximately 300 mOsm/kg (range 296–314 mOsm/kg); pH 7.25 with or without agonists/antagonists.

Reverse transcriptase-PCR analyses

Reverse transcriptase-PCR analysis of single JG cells and preglomerular vessels was as described.[19] Primer sequences: CnA-α: sense 5′-CAA-GAT-CCG-AGC-AAT-AGG-3′ antisense 5′-TCC-TTT-GAT-GGC-TTC-ATC-3′; CnA-β: sense 5′-GGA-TGG-ATT-AGC-ATG-GTC-3′ antisense 5′-TAG-AAA-TAG-TTT-ATG-CAT-3′; CnA-γ: sense 5′-CTG-CTC-CTA-CTT-CTT-CAG-3′ antisense 5′-GAA-AGC-CAG-TTG-CTT-GGT-3′; Cyclophilin: sense 5′-AAG-CCA-TGG-AGC-GTT-TTG-3′ antisense 5′-CTT-CAG-TGA-GAG-CAG-AGA-3′; FKBP12: sense 5′-TTG-ACT-CCT-CTC-GGG-ACA-3′ antisense 5′-CAT-AGT-CTG-GGG-AGA-TTA-3′. Actin primers were as described.[19] Amplification products were sequenced using the MWG sequencing service (Ebersberg, Germany). For quantitative PCR, RNA was isolated from kidney tissue using a kit (Qiagen, Copenhagen, Denmark) and cDNA was prepared as previously described.[32] Human brain RNA (20 μg) was purchased from Ambion (Applied Biosystems, Naerum, Denmark). An iQ-Thermocycler (Bio-Rad, Copenhagen, Denmark) was used as described.[32] Renin primers: sense 5′-GCT-ATG-TGA-AGA-AGG-CTG-3′ and antisense 5′-TTC-TCT-TCT-CCT-TGG-CTC-3′. Renin concentration was determined as previously described.[32,33]

Agents

4,(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES); Tris-HCl; glucose, sucrose, insulin, penicillin, streptomycin, K-glutamate, magnesium-adenosine triphosphate, forskolin, dithiothreitol, trypsin, EGTA, CsA, tacrolimus, calmidazolium chloride, and calcineurin-inhibitory peptide were from Sigma Chemical (St Louis, MO, USA). RPMI 1640 and fetal calf serum were from GIBCO Life Technologies (Invitrogen, Taastrup, Denmark). Collagenase A, Na_2-GTP, and cAMP were from Roche (Hvidovre, Denmark). RpcAMPs were from Biomol (Plymouth Meeting, PA, USA) and W-13 was from VWR (Bie & Berntsen, Herlev, Denmark). Stock solutions of ligands were made in ethanol or dimethyl sulfoxide and kept at −20 °C.

Statistics

The effect of a perturbation on C_m was determined as the difference between C_m at $t = 0$ min and $t = 10$ min (ΔC_m) and the null hypothesis was that $\Delta C_m = 0$. This was tested using paired Student's t-test. There were no differences in mean C_m at $t = 0$ between the eight experimental series in the study and data are presented as mean $\Delta C_m \pm$ s.e. in percentage of C_m at $t = 0$. Unpaired Student's t-test was used to compare means of two groups. Analysis of variance was used to determine whether there was statistical significance among several groups of data in renin secretion data. Dunnett's test was performed *post hoc* to test for significant differences between experimental groups and control group. A $P < 0.05$ was considered statistically significant. Calculations were performed using GraphPadPrism software (La Jolla, CA, USA).

DISCLOSURE

All the authors declared no competing interests.

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